REGULATION STUDIES OF POLYHYDROXYALKANOATE SYNTHASE GENES IN *Pseudomonas* sp. USM 4-55

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REGULATION STUDIES OF POLYHYDROXYALKANOATE SYNTHASE GENES IN *Pseudomonas* sp. USM 4-55

by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
°C	degree Celsius
α	alpha
atm	standard atmospheric pressure
ADP	adenosine diphosphate
ATP	adenosine triphosphate
β	beta
bp	base pair
BLAST	Basic Local Alignment Search Tool
Δ	delta
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
g	gram
kb	kilo base pair
kPa	kilo pascal
L	liter
M	molar
mg	miligram
min	minute
ml	milliliter
mM	milimolar
mRNA	messenger ribonucleic acid

ng	nanogram
psi	pound per square inch
pg	picogram
RNA	ribonucleic acid
rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
σ	sigma
sec	second
SDS	sodium dodecyl sulphate
siRNA	small interference ribonucleic acid
sp./spp.	species
SSC	standard saline buffer
tRNA	transfer ribonucleic acid
U	unit
µg	microgram
µl	microliter
µM	micromolar
wt	weight
w/v	weight per volume
V	volt
v/v	volume per volume
ω	omega

KAJIAN PENGAWALATURAN GEN-GEN POLIHIDROKSIALKANOAT SINTASE DALAM *Pseudomonas* sp. USM 4-55

ABSTRAK

Polihidroksialkanoat (PHA) merupakan satu kelas poliester yang wujud secara semulajadi dan berfungsi sebagai bahan penyimpan tenaga secara intrasel untuk memastikan kemandirian bakteria dalam keadaan yang ekstrem. Kuantifikasi relatif transkrip mRNA gen-gen *phaC1*, *phaC2* dan *phbC* dalam *Pseudomonas* sp. USM 4-55 dikaji dengan menggunakan teknik kuantitatif transkripsi berbalik-PCR masa nyata (RT-qPCR). Transkrip gen *phbC* adalah paling banyak diikuti oleh gen *phaC1* dan seterusnya gen *phaC2* apabila dikultur atas asid oleik, PFAD atau glukosa sebagai sumber karbon tunggal semasa fasa log dan fasa pegun. Transkrip gen *phaC1* adalah sepuluh kali atau lebih berbanding dengan transkrip gen *phaC2*. Dalam kultur PFAD, penghasilan PHA yang paling tinggi direkod ialah 50 wt% dengan 66 mol% monomer berkarbon sederhana semasa fasa log lewat; manakala glukosa menghasilkan PHA tertinggi sebanyak 30 wt% dengan 72 mol% 3HB (monomer berkarbon pendek) semasa fasa pegun. Teknik RT-qPCR dan PCR transkripsi berbalik (RT-PCR) menunjukkan bahawa gen *phaC1* dan *phaC2* adalah tidak ditranskripsikan bersama dan mempunyai promoter tersendiri manakala gen *phbC* ditranskripkan sebagai satu operon *phbBAC* dan dikawal oleh satu promoter yang aktif walaupun semasa fasa pegun. Ramalan tapak promoter dengan menggunakan BPROM dan PromScan menunjukkan bahawa terdapat satu tapak promoter σ^D- dan satu tapak promoter σ^N-bersandar yang terletak pada 206 bp dan 125 bp di hilir gen *phaC1* masing-masing. Pemetaan hujung 5’ gen *phaC2* dengan menggunakan teknik

cRACE mendedahkan dua tapak permulaan transkripsi putatif pada 39 bp di hilir kodon permulaan putatif pertama dan pada 93 bp di hilir kodon permulaan putatif kedua. Teknik CR-RT-PCR mengesahkan tapak permulaan transkripsi putatif yang pertama iaitu terletak pada 39 bp di hilir kodon permulaan putatif pertama. Dua jujukan promoter, iaitu σ^N- dan σ^D-bersandar juga didapati pada 145 bp di hilir ORF rujukan dan pada 61 bp di hilir ORF putatif pertama gen *phaC2* masing-masing. Hanya satu tapak promoter σ^D-bersandar diperolehi pada 175 bp di hilir operon *phbBAC*.

REGULATION STUDIES OF POLYHYDROXYALKANOATE SYNTHASE GENES IN *Pseudomonas* sp. USM 4-55

ABSTRACT

Polyhydroxyalkanoate (PHA) is a class of naturally occurring polyesters that serve as intracellular energy storage material for bacteria that facilitates its survival during extreme environmental conditions. The relative quantification of mRNA transcripts of three PHA synthase genes, *phaC1*, *phaC2* and *phbC* in *Pseudomonas* sp. USM 4-55 was examined utilizing quantitative reverse transcription-real time PCR (RT-qPCR). The transcript level of *phbC* was the most abundant followed by *phaC1* and then *phaC2* when grown on either oleic acid, PFAD or glucose as the sole carbon source during log and stationary phase. The transcript level of *phaC1* was found to be ten times higher or more than the transcript level of *phaC2*. In PFAD culture, the highest recorded PHA production was 50 wt% with 66 mol% of mcl monomers during the late log phase; while glucose produced highest PHA of 30 wt% with 72 mol% of 3HB (scl) monomer during the stationary phase. The RT-qPCR and the reverse transcription PCR (RT-PCR) revealed that *phaC1* and *phaC2* are not cotranscribed and possess their own promoters while the *phbC* is transcribed as the *phbBAC* operon and controlled by a single promoter which is active even during stationary phase. Promoter prediction using BPROM and PromScan disclosed a σ^D- and a σ^N-dependent promoter region at 206 bp and 125 bp upstream of *phaC1* respectively. The 5' termini mapping of *phaC2* using cRACE method revealed two putative transcriptional start sites 39 bp upstream of the first putative start codon and 93 bp upstream of the second putative start codon. The CR-RT-PCR method

confirmed the first putative transcriptional start site located 39 bp upstream of the first putative start codon. Two promoter regions, σ^N- and σ^D-dependent promoter sequences were detected 145 bp upstream of reference ORF and 61 bp upstream of the first putative ORF of *phaC2*, respectively. Only a σ^D-dependent promoter region was found 175 bp upstream of operon *phbBAC*.

CHAPTER 1 GENERAL INTRODUCTION

The last few decades has witness the quantum leap of science and technology in aiding everyday life. However, such enormous progress has also brought with it various problems, one of which is solid waste disposal especially plastics that can remain forever.

Bioplastics can serve as a solution for solving the major solid waste disposal problem because it is fully biodegradable. Biodegradable plastics can also contribute as an alternative energy source to the depleting fossil resources.

Today, one of the most promising bioplastics is polyhydroxyalkanoate (PHA) which is produced and accumulated by bacteria as a carbon and energy reserve material. It possesses potential applications in various fields including packaging, medical, pharmaceutical, food, agriculture and others (Khanna & Srivastava, 2005; Sudesh & Iwata, 2008). It is considered as an environmental-friendly plastic and provides a substitution for the synthetic non-biodegradable plastics. PHA is unique due to its ability of being synthesized from renewable resources and their complete biodegradation into water and carbon dioxide (Jendrossek, 2001; Jendrossek & Handrick, 2002; Khanna & Srivastava, 2005; Prieto *et al.*, 2007; Sudesh & Iwata, 2008).

Since the discovery of PHA, a lot of studies have been conducted to fully understand this natural polymer. All these efforts were to enable wide utilization of PHA and the replacement of non-biodegradable synthetic plastic. The biggest obstacle that prevents wide-scale application of PHA is the high cost of large scale production (Lenz & Marchessault, 2005; Verlinden *et al.*, 2007). Studies to surmount

this challenge include utilization of cheap substrates, utilization of various recombinant strains and modification of the pathway-associated enzymes to improve its material properties, increasing productivity, thus leading to reduction in the cost of production.

PHA can be classified into two major groups, namely short-chain-length (scl-) PHA and medium-chain-length (mcl-) PHA. Various types of bacteria were found to accumulate PHA as carbon and energy storage. The key enzyme in producing PHA is PHA synthase. The study carried out here focus on the gene expressing PHA synthase in a bacterium named *Pseudomonas* sp. USM 4-55.

The bacterium *Pseudomonas* sp. USM 4-55 was discovered in a soil sample taken from an oil palm plantation in Tasek Chini, Pahang, Malaysia by Anthoni Augustien (Razip Samian, personal communication). The uniqueness of this bacterium is that it possesses three PHA synthase genes, namely *phaC1*, *phaC2* and *phbC*. The presence of these genes enables it to accumulate a blend of P(3HB) (poly-3-hydroxybutyrate, a scl-PHA) and mcl-PHA homopolymer, and a minute amount of random copolymer [P(3HB-co-3HA)] (Sudesh *et al.*, 2004). The *phaC1* and *phaC2* are believed to be responsible for mcl-PHA synthesis while the *phbC* was proven to be active towards scl-PHA production.

The *pha* gene cluster consist of *phaC1*, *phaZ* and *phaC2* (*phaZ* is a depolymerase gene) (Baharuddin, 2002). On the other hand, the operon *phb* is organized in an order of *phbB*, *phbA* and *phbC* (*phbB* is NADPH-dependent acetoacetyl-coenzyme A reductase and *phbA* is β-ketothiolase) (Tan *et al*., 2010). To date, there is another bacterium reported, *Pseudomonas* sp. 61-3 that demonstrated the same type of PHA accumulation ability and the same genetic organization of genes (Matsusaki *et al.*, 1998). Other pseudomonads reported to be able to produce

mixture of scl- and mcl-PHA are *Pseudomonas oleovorans* strain B-778 (Ashby *et al.*, 2002), *Pseudomonas stutzeri* 1317 (Chen *et al.*, 2006), and *Pseudomonas* sp. LDC-5 (Ashby *et al.*, 2002; Chen *et al.*, 2006; Sujatha *et al.*, 2007). Other pseudomonads are only able to accumulate mcl-PHA solely, i.e *Pseudomonas aeruginosa* PAO1 (Timm & Steinbuchel, 1992), *Pseudomonas mendocina* (Hein *et al.*, 2002), *Pseudomonas corrugata* (Solaiman *et al.*, 2002) and *Pseudomonas putida* KT2442 (Addison *et al.*, 2007; Huijberts *et al.*, 1992).

The fact that *Pseudomonas* sp. USM 4-55 possesses three PHA synthase genes has sparked the idea of studying the regulation of these genes in various conditions. Through regulation studies, the gene expression can be correlated with the PHA production. These provide a better understanding of the PHA synthase genes and lay a foundation for future recombinant technology manipulation of these enzymes and in this bacterium.

Not many studies about regulation has been conducted, and this is the first ever regulation study about a *Pseudomonas* strain with three PHA synthases. The objectives of this study are:

1) To determine the transcript levels of these three PHA synthase genes (*phaC1*, *phaC2* and *phbC*)
2) To characterize the regulatory elements of these three PHA synthase genes

The regulation of these genes was investigated from a few angles in this study, namely scrutinizing each gene expression level with the PHA production, examined the transcriptional profile through the mRNA transcript and the promoter regions, and finally looking deeper into the transcriptional start site of each gene in order to confirm the authentic transcripts expressed. To fully discuss the experiments

conducted, this study was briefly divided into three major chapters, namely gene expression level and PHA accumulation study, mRNA transcript analysis and 5' termini mapping of mRNA transcript.

CHAPTER 2 LITERATURE REVIEW

2.1 POLYHYDROXYALKANOATE (PHA)

2.1.1 PHA as storage material

Polyhydroxyalkanoates (PHAs) are a group of polyesters synthesized by most genera of bacteria and members of the family *Halobacteriaceae* of the Archaea (Steinbuchel *et al.*, 1995). Bacteria synthesize this polymer and store it as an intracellular carbon and energy material. When the environment of bacteria has excess carbon sources and limited amounts of nutrients, such as nitrogen, phosphate, or magnesium, the bacteria will then start accumulating PHA (Anderson & Dawes, 1990; Doi, 1990).

Bacteria can accumulate as much as 90% of the cell dry weight of PHA which are usually stored as granules (Madison & Huisman, 1999). The production of PHA occurs in different phases of growth depending on different bacteria in different environments. The accumulation of PHA gives the advantage to the bacteria for survival in starvation or extreme environments (Kadouri *et al.*, 2005; Zhao *et al.*, 2007). The bacteria will then synthesize intracellular or extracellular depolymerase enzymes to degrade this storage compound and utilize it as carbon and energy source (Philip *et al.*, 2007). Synthesizing PHA also maintains the osmotic state and prevents compounds leakage out of the cell as this is a process of changing soluble intermediates into insoluble intermediates (Madison & Huisman, 1999).

2.1.2 History of PHA discovery

The ability of producing polyesters by bacteria was unknown to many scientists before the 1960s. However, the truth is, these polymers were first

discovered in 1845 (Sudesh & Iwata, 2008) and even studied and characterized in the literature as early as 1926, by a French bacteriologist, Maurice Lemoigne. Lemoigne was the first to identify the inclusions in *Bacillus megaterium* as poly(3-hydroxybutyric acid) [P(3HB)] (Lemoigne, 1923). At that time, Lemoigne published his discovery in a series of little-read French journals which were not discovered till the late 1950s. Lemoigne and co-workers also found that *B. megaterium* could contain as much as 44% of their cell dry weight of P(3HB) depending on the growth conditions (René, 1967). Lemoigne also described an analytical method for quantifying P(3HB) and showed that P(3HB) could be cast into a transparent film (Lemoigne, 1926).

In 1974, Wallen and Rohwedder reported other compositions of polyester apart from P(3HB) (Wallen & Rohwedder, 1974). They found both 3HB and also 3-hydroxyvalerate (3HV) in extracts of activated sludge. In 1983, White and co-workers analyzed bacteria extract from marine sediments with gas chromatography and found at least 11 types of repeating units, including both linear and branched 3HA with compositions varying from 4-8 carbon atoms. They also showed that Lemoigne's original bacteria, *B. megaterium* could produce polymers which contained 95% of 3HB, 3% of 3-hydroxyheptanoate (3HHp), 2% of 3-hydroxyoctanoate (3HO) and trace amounts of three other 3HA components (Findlay & White, 1983). In the same year, Witholt and co-workers discovered that *Pseudomonas oleovorans* which was grown on alkanes could produce polymers consisting principally 3HO and small amounts of 3-hydroxyhexanoate (3HHx) units (de Smet *et al*, 1983, Lenz & Marchessault, 2005; Sudesh *et al.*, 2000).

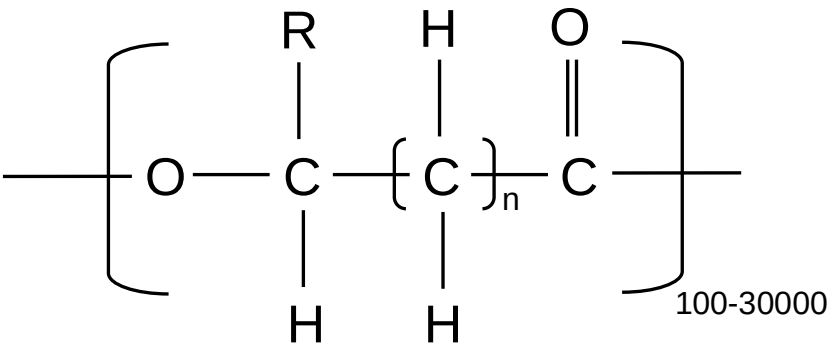
2.1.3 Types of PHA

The monomer units of PHAs contain (R)-3-hydroxyalkanoic acid (Anderson & Dawes, 1990). The general chemical structure of PHA is depicted in Figure 2.1. More than 150 different monomers have been detected to date in which the units range from 3-hydroxypropionic acid to 3-hydroxyhexadecanoic acids (Chen & Wu, 2005a; Rehm, 2007). PHA monomers with different types of side chain, which include straight, branched, saturated, unsaturated and aromatic were identified (Steinbuchel & Valentin, 1995). Functional groups in the side chains which comprise of halogens, hydroxy, carboxy, epoxy, phenoxy, cyanophenoxy, nitrophenoxy, thiophenoxy, and methyl ester groups are of interest as it allow further chemical modifications to improve the physical properties (Hazer & Steinbuchel, 2007).

In general, short-chain-length PHAs (scl-PHAs) contain 3-5 carbon atoms in the monomer units, while medium-chain-length PHAs (mcl-PHAs) contain 6-14 carbon atoms in the monomer units (Steinbuchel *et al.*, 1992). Bacteria produce various PHAs that are structurally related to the monomer constituents based on different fatty acids or aliphatic carbon precursor substrates that are being fed. There is another type of copolymer PHA which consists of both short-chain-length and medium-chain-length PHAs (scl-mcl-PHA), containing 3-14 carbon atoms (Matsusaki *et al.*, 1998; Steinbuchel & Hein, 2001).

2.1.4 Physical properties of PHA

The molecular mass of PHAs is about 50,000 to 1,000,000 Daltons (Da) varies upon growth conditions, carbon sources, types of PHA synthases and also metabolic pathways that are active in the cell (Sudesh & Iwata, 2008). These high molecular mass have parallel characteristic as conventional plastics such as polypropylene (Madison & Huisman, 1999).



n = 1	R = hydrogen	poly(-3-hydroxyproprionate)
	methyl	poly(-3-hydroxybutyrate)
	ethyl	poly (-3-hydroxyvalerate)
	propyl	poly(-3-hydroxyhexanoate)
	pentyl	poly(-3-hydroxyoctanoate)
	nonyl	poly(-3-hydroxydodecanoate)
n = 2	R = hydrogen	poly(-4-hydroxybutyrate)
n = 3	R = hydrogen	poly(-5-hydroxyvalerate)

Figure 2.1 The chemical structure of PHA (adapted from Ojumu *et al*., 2004).

PHAs exist in a form of water-insoluble inclusions and are amorphous in the bacterial cell. After extraction out of the cell with organic solvent, PHAs crystallize rapidly (Barnard & Sanders, 1989). The degree of crystallinity falls between 60-80% (Sudesh *et al.*, 2000). Table 2.1 shows the comparison of properties between PHAs with other plastics.

Scl-PHAs have high crystallinity and thus appear as a stiff but brittle material after storage under ambient conditions for several days. Due to this, it is not very stress resistant. It also possesses a high melting temperature which is around 180°C that limits its ability to be processed (Madison & Huisman, 1999; Ojumu *et al.*, 2004).

On the other hand, mcl-PHAs are more elastomeric and sticky which possess a low degree of crystallinity and a low melting temperature (Kim *et al.*, 2007). The polymer becomes softer as the monomer chain length increases. The characteristic of mcl-PHAs is largely influenced by the length and types of groups in the side chain (Zinn *et al.*, 2001).

Many researches has been carry out to understand the brittle nature of P(3HB) and also to improve the physical properties of this polymer. One of the idea is to incorporate other HAs into P(3HB) to form copolymers so that the molecular weight, crystallinity, stiffness and toughness can be improved (Khanna & Srivastava, 2005). One of the example is the incorporation of 3HV into P(3HB), which results in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)], a random copolymer that is less stiff, more flexible and tougher as compared to P(3HB). This copolymer can be used to prepare films with excellent water and gas barrier properties reminiscent of polypropylene. Moreover, it can be processed at a lower temperature while maintaining most of the other mechanical properties of P(3HB)

Table 2.1 Comparison of physical properties of various PHAs to other commercial plastics (adapted from Khanna & Srivastava, 2005; Ojumu *et al*., 2004; Verlinden *et al*., 2007).

Polymer	Melting temperature (°C)	Glass-transition temperature (°C)	Tensil Strength (MPa)	Young's Modulus (GPa)	Elongation to break (%)
P(3HB)	180	4	40	3.5	5
P(4HB)	53	-	104	149	1000
P(3HB-co-6 mol% 3HA)	133	-8	17	0.2	680
P(3HB-co-3HV)					
3 mol% 3HV	170	-	38	2.9	-
9 mol% 3HV	162	-	37	1.9	-
20 mol% 3HV	145	-1	20	0.8	50
71 mol% 3HV	83	-13	-	-	-
P(3HB-co-3HHx)	52	-4	20	-	850
P(3HHx-co-3HO)	61	-	10	-	300
P(3HB-co-4HB)					
3 mol% 4HB	166	-	28	-	45
16 mol% 4HB	150	-7	26	-	444
90 mol% 4HB	50	-	65	100	1080
Polypropylene	176	-10	38	1.7	400
Low-density polyethylene	130	-30	10	0.2	620
Polystyrene	110	21	50	3.1	-

(Marchessault, 1996; Tanaka *et al.*, 2006). Incorporation of other HA units into P(3HB) will yield a enormous range of different copolymers harvesting different properties which will be useful in a wider range of applications.

2.1.5 Biological properties and biodegradation

The combination of genetic engineering, modern molecular microbiology and biological polymerization systems has enabled the generation of high-molecular weight materials using PHAs as raw material and has resulted in an enormous range of new polymers (Madison & Huisman, 1999).

The most important characteristic of PHA is its complete biodegradability. PHAs will go through biodegradation after being disposed into a microbially active environment. Biodegradation can occur in sea water, soil, lake water, aerobic or anaerobic conditions etc. The rate of degradation also differs depending on different environmental conditions such as pH, moisture level, temperature, nutrient supply and other factors (Khanna & Srivastava, 2005). The degradation rate of a piece of P(3HB) can span across a few months (in anaerobic sewage) to years (in seawater) (Jendrossek *et al.*, 1996; Mergaert *et al.*, 1993; Mergaert *et al.*, 1995; Mergaert *et al.*, 1996; Ojumu *et al.*, 2004) and even days when higher activities of microbial colonies occurred (Sudesh & Iwata, 2008). Copolymers tend to degrade faster than homopolymers. Crystallinity has been reported to affect the biodegradation rate significantly especially in P(3HB-co-3HV) (Kim *et al.*, 2004).

Bacteria will colonize the surface of PHA and secrete an extracellular depolymerase to degrade the polymer into its monomer units. The soluble degraded products will then be absorbed through the cell wall and metabolized in the cell. The end products of biodegradation are carbon dioxide (CO_2) and water in aerobic conditions while methane and CO_2 are released in anaerobic conditions. Storage

PHAs also can be degraded by intracellular PHA depolymerase enzyme in a mechanism known as PHA mobilization (Jendrossek *et al.*, 1996).

The fact that PHAs can be produced from renewable resources is one of the important characteristics of this biopolymer. Unlike the production of polypropylene from petroleum, PHAs production is based on agricultural products such as sugar and fatty acids as carbon and energy sources. These provide CO_2 and water, and after became PHAs, the biodegradation of PHAs will again produce CO_2 and water (Madison & Huisman, 1999; Sudesh & Iwata, 2008).

2.1.6 Applications and future of PHAs

PHAs have great advantages over petrochemical polymers mainly because of its biodegradability and their other novel features. PHAs can also be partially digested and then recombined with other polymers to produce other products with specific properties. The mixture changes the plastic crystallinity and also the crystallization rate, besides changing the mechanical properties of the material (Zinn *et al.*, 2001).

In 1982, Imperial Chemical Industries Ltd. (ICI) in England commercially produced a biodegradable thermoplastic polyester for packaging applications under the trade name Biopol^{TM}. This first type of commercialized PHA was a random copolymer consisted of P(3HB-co-3HV). A biosynthesis process was developed utilizing *Cupriavidus necator* (formerly *Wautersia eutropha*, *Ralstonia eutropha* and *Alcaligenes eutrophus*) to produce P(3HB-co-3HV) (Lenz & Marchessault, 2005; Sudesh & Iwata, 2008).

PHAs are used in packaging films mainly in bags, containers and paper coatings. They can also be used in conventional commodity plastics area such as

disposable items like razors, utensils, diapers, feminine hygiene products, cosmetic containers, shampoo bottles and cups (Khanna & Srivastava, 2005).

PHAs also been processed into fibers which will then be used to construct materials such as nonwoven fabrics, used as hot-melt adhesives, toner and developer compositions, ion-conducting polymers, dairy cream substitutes or flavor delivery agents in foods (Madison & Huisman, 1999).

In the medical area, PHAs attract great attention as the biodegradable plastic can be inserted into the human body and need not be removed. P(3HB) is also a normal constituent of the blood at concentrations between 0.3 and 1.3mM (Zinn *et al.*, 2001). PHAs are used as osteosynthetic materials in the stimulation of bone growth, in bone plates, surgical sutures and blood vessel replacements (Khanna & Srivastava, 2005; Steinbuchel & Fuchtenbusch, 1998; Verlinden *et al.*, 2007). PHAs also function as a matrix in retardant materials for the slow release of drugs, hormones, herbicides and insecticides (Chen & Wu, 2005b; Steinbuchel & Fuchtenbusch, 1998). Lately, electrospun PHAs proved to be a promising biomaterial in tissue-engineering scaffolds that can be tailored-made according to various needs (Ying *et al.*, 2008).

PHAs can also be produced as tailor-made bioparticles, which can be applied for drug delivery, diagnostics, bioseparation, protein immobilization etc. (Brockelbank *et al.*, 2006; Peters & Rehm, 2006; Rehm, 2006). The size, surface functionality, and core composition can be highly controlled and thus can provide a platform technology for the production of functionalized, biocompatible and biodegradable nanoparticles (Rehm, 2007). Besides, these bioparticles can be used to display proteins or for protein production (Barnard *et al.*, 2005; Brockelbank *et al.*, 2006; Rehm, 2006).

PHAs also give a hope to function as new source of small molecules. PHA can be hydrolyzed chemically, thus turning the monomers into commercially attractive molecules such as β-hydroxy acids, 2-alkenoic acids, β-hydroxyalkanols, β-amino acids etc.(Williams & Peoples, 1996).

2.2 STRUCTURE OF PHA GRANULES

PHA granules appear as a water-insoluble spherical inclusions surrounded by a phospholipid membrane (Rehm, 2006) with embedded or attached proteins (Stuart *et al.*, 1998). This includes PHA synthase (Gerngross *et al.*, 1993), intracellular PHA depolymerase (Handrick *et al.*, 2000), amphipathic phasin proteins (Pieper-Furst *et al.*, 1995), PHA-specific regulator proteins (Maehara *et al.*, 2002; Prieto *et al.*, 1999; York *et al.*, 2002) and additional proteins (Klinke *et al.*, 2000) with unknown function.

2.3 *IN VIVO* POLYESTER PARTICLES FORMATION

In vivo PHA synthesis starts as soon as the substrate, (*R*)-3-hydroxyacyl-CoA thioesters is available intracellularly. Naturally, PHA synthase is produced constitutively in low amounts but begin to catalyze high molecular weight polymer upon availability of substrates (Rehm, 2007).

Currently there are two models of PHA granule formation, the micelle model and the budding model. The models are shown in Figure 2.2. The micelle model supports by PHA granule formation *in vitro*. In the absence of membranes, (*R*)-3-hydroxyacyl-CoA will attach to free soluble PHA synthase and convert it into amphipathic PHA synthase which will then form insoluble polyester particles.

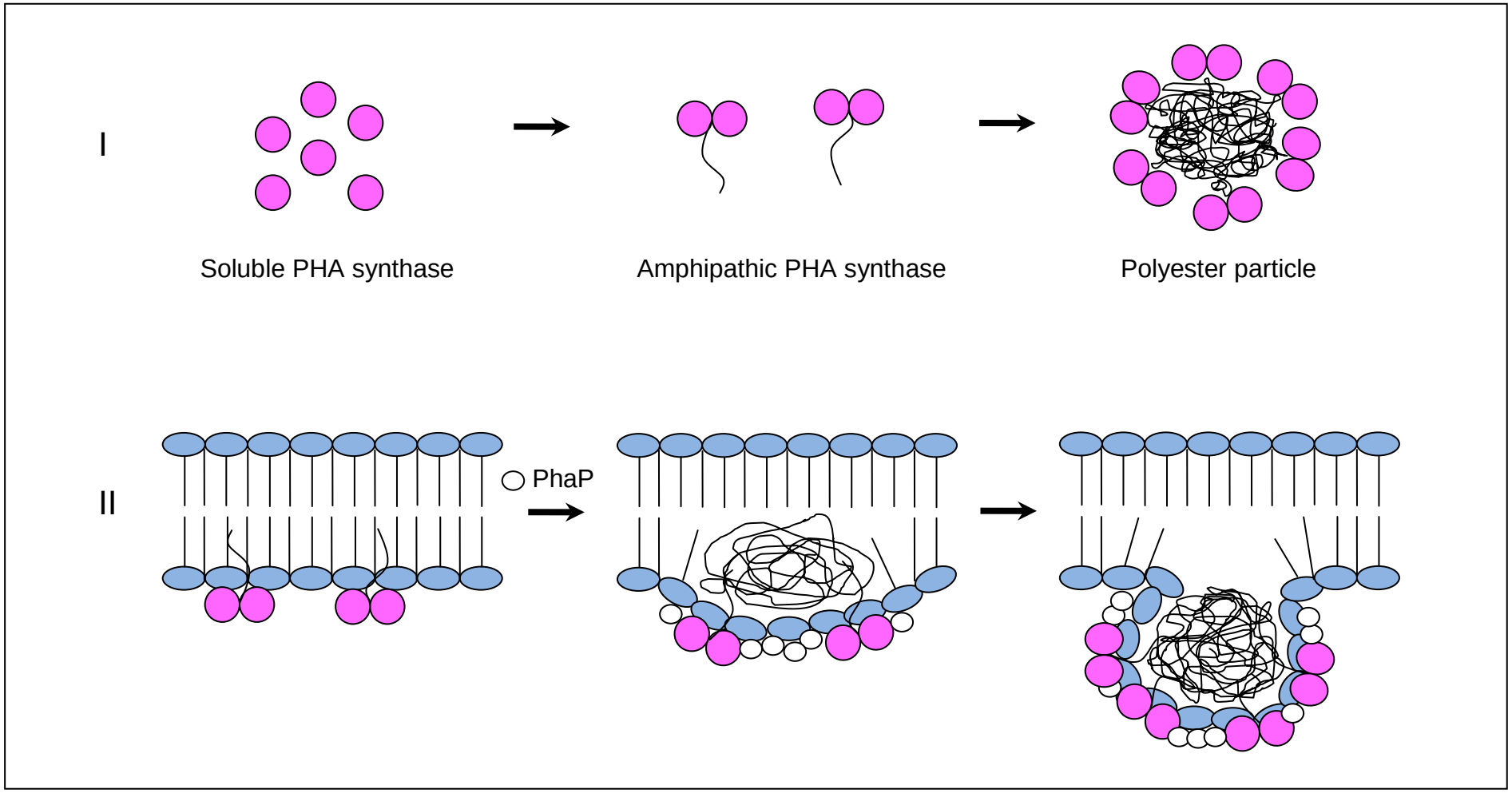


Figure 2.2 PHA granule formation models. (I) Micelle model representing *in vitro* formation in the absence of membranes and (II) budding model representing granule formation at the cytoplasmic membrane (adapted from Rehm, 2007; Tian *et al*., 2005).

According to the budding model, the hydrophobic PHA synthase binds to the inner face of the plasma membrane, accumulates and then buds out before it is being released into the environment. This leads to a granule surface which is covered with a lipid monolayer (Tian *et al.*, 2005). In a study which utilized green fluorescent protein (GFP)-labeled PHA synthase, the early stage granules were found to be localized at the cell poles and were dependent on nucleoid structure. This study also showed that small emerging granules oscillate between the cell poles and thus contribute to the equal distribution of storage materials between the daughter cells (Peters & Rehm, 2005).

In both models, the defined location of the PHA synthase and phasin protein on the surface of the granule are taken into consideration (Rehm, 2007). Both models reveal some common points. Firstly, the soluble PHA synthase is being converted into a amphipathic compound during polyester chain synthesis. Secondly, the process of self-assembly occurs either in the membrane or in the cytosol (Rehm, 2007). During the self-assembly process, spherical and water-insoluble inclusions are formed with an amorphous polyester core and PHA synthase is covalently bound to the surface (Gerngross *et al.*, 1993; Mayer & Hoppert, 1997). The bound PHA synthase will continuously convert the precursor from the cytosol and then provide the substrate to the continually growing polyester chain in increasing the PHA granule size (Rehm, 2007).

2.4 PHA GRANULE FORMATION IN *Pseudomonas* sp. USM4-55

The PHA granules of *Pseudomonas* sp. USM4-55 have been isolated and studied in detail. The micelle model and the budding model cannot be used to explain the results obtained for the granule formation of *Pseudomonas* sp. USM4-55. Thus, a

different mechanism was adopted to discuss the PHA granule formation event in *Pseudomonas* sp. USM4-55.

Pseudomonas sp. USM4-55 is capable of producing a blend of P(3HB) homopolymer and mcl-PHA that also contains small amount of 3HB monomers. The latter 3HB monomers are most possibly copolymerized with the mcl-PHA as they could not be separated with solvent fractionation. The P(3HB) granules and the mcl-PHA granules exist in the same cell but present separately as different granules. This indicated that *Pseudomonas* sp. USM4-55 possesses another PHA synthase which is specific towards scl-PHA (Tan *et al.*, 2010). The scl-PHA synthase and mcl-PHA synthase have broad substrate specificity and thus do not produce single PHA granules (Sudesh *et al.*, 2004).

Two mechanisms were suggested to explain the granule formation in *P.* USM4-55 and depicted in Figure 2.3. In the first mechanism, the segregation of the two types of PHA into different granules may due to phasin proteins that can directly interact with the hydrophobic PHA chains (Sudesh *et al.*, 2002; Sudesh *et al.*, 2004). In the second mechanism, the formation of PHA synthase complex is due to the observation of a distinct lag phase in the polymerization reaction catalyzed by the enzyme. The enzymes with similar substrate specificity will form a complex with each other and thus each of the PHA synthase will produce one granule (Sudesh *et al.*, 2004). It should be noted that this mechanism is also supported by the theory of chain transfer reaction for the polymerization of PHA by the PHA synthase (Kawaguchi & Doi, 1992).

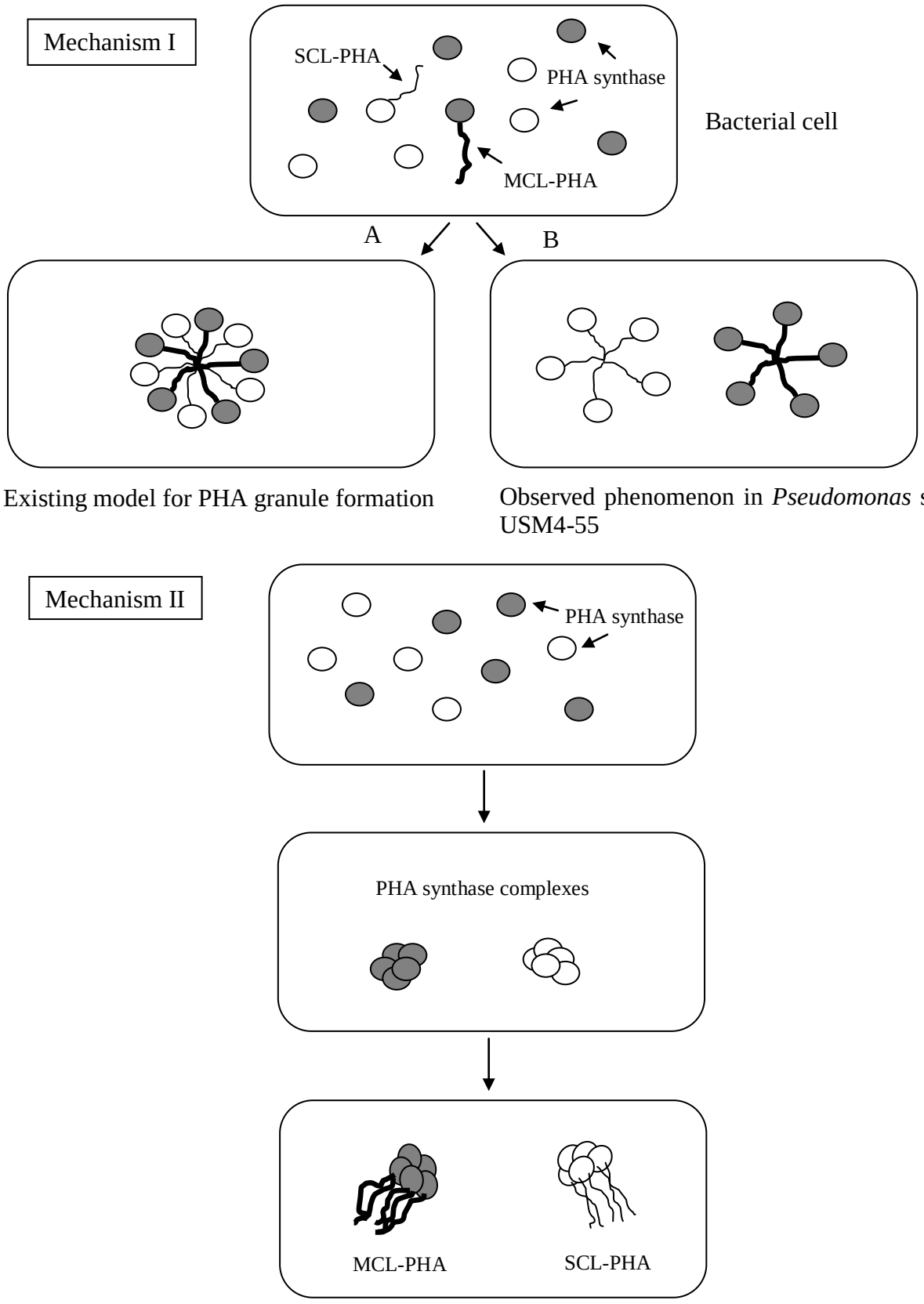


Figure 2.3 Predicted granule formation model in *Pseudomonas* sp. USM4-55. White spheres represent scl-PHA synthase while grey spheres represent mcl-PHA synthase. Thin lines represent growing scl-PHA chains while thick lines represent growing mcl-PHA chains that also contain small amounts of 3HB monomer. Mechanism I(A) shows existing model for PHA formation. Mechanism I(B) shows the observed phenomenon in *Pseudomonas* sp. USM4-55. Mechanism II illustrates the proposed new model for PHA granule formation in *Pseudomonas* sp. USM4-55 (adapted from Sudesh *et al*., 2004).

2.5 BIOSYNTHESIS AND METABOLIC ROUTES OF POLYHYDROXYALKANOATES

PHAs can be categorized into two different classes based on their compositions. First, PHAs consisting short-chain-length 3-hydroxy fatty acids, ranging from 3-5 carbon atoms, also known as P(3HB)/PHB is represented by a classic P(3HB) producer, *Cupriavidus necator*. Second, PHAs consisting medium-chain-length 3-hydroxy fatty acids, ranging from 6-14 carbon atoms, is represented by pseudomonads (Rehm, 2007). In general, PHA synthases show very broad substrate specificity and can polymerize a wide variety of monomers. Figure 2.4 summarizes the various major metabolic routes for PHA biosynthesis.

2.5.1 Biosynthesis of scl-PHA

2.5.1.1 Pathway I

The biosynthesis pathway of PHB has been well studied in *C. necator*. Metabolism of carbohydrates leads to the biosynthesis of PHA (Naik *et al.*, 2008). The biosynthesis process begins with condensation of two acetyl-CoA molecules to acetoacetyl-CoA by enzyme β-ketothiolase (PhaA). NADPH-dependent acetoacetyl-CoA reductase (PhaB) will then function to reduce Acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA. Finally PHA synthase (PhaC) will polymerize the (*R*)-3-hydroxybutyryl-CoA and produce PHB (Rehm, 2007; Sudesh *et al.*, 2000).

2.5.2 Biosynthesis of mcl-PHA

2.5.2.1 Pathway II

This pathway is also known as the *de novo* fatty acid biosynthesis pathway, and is involved in mcl-PHA synthesis when gluconate or other non-related carbon sources (carbohydrates) are used. The important enzyme here is 3-hydroxylacyl-

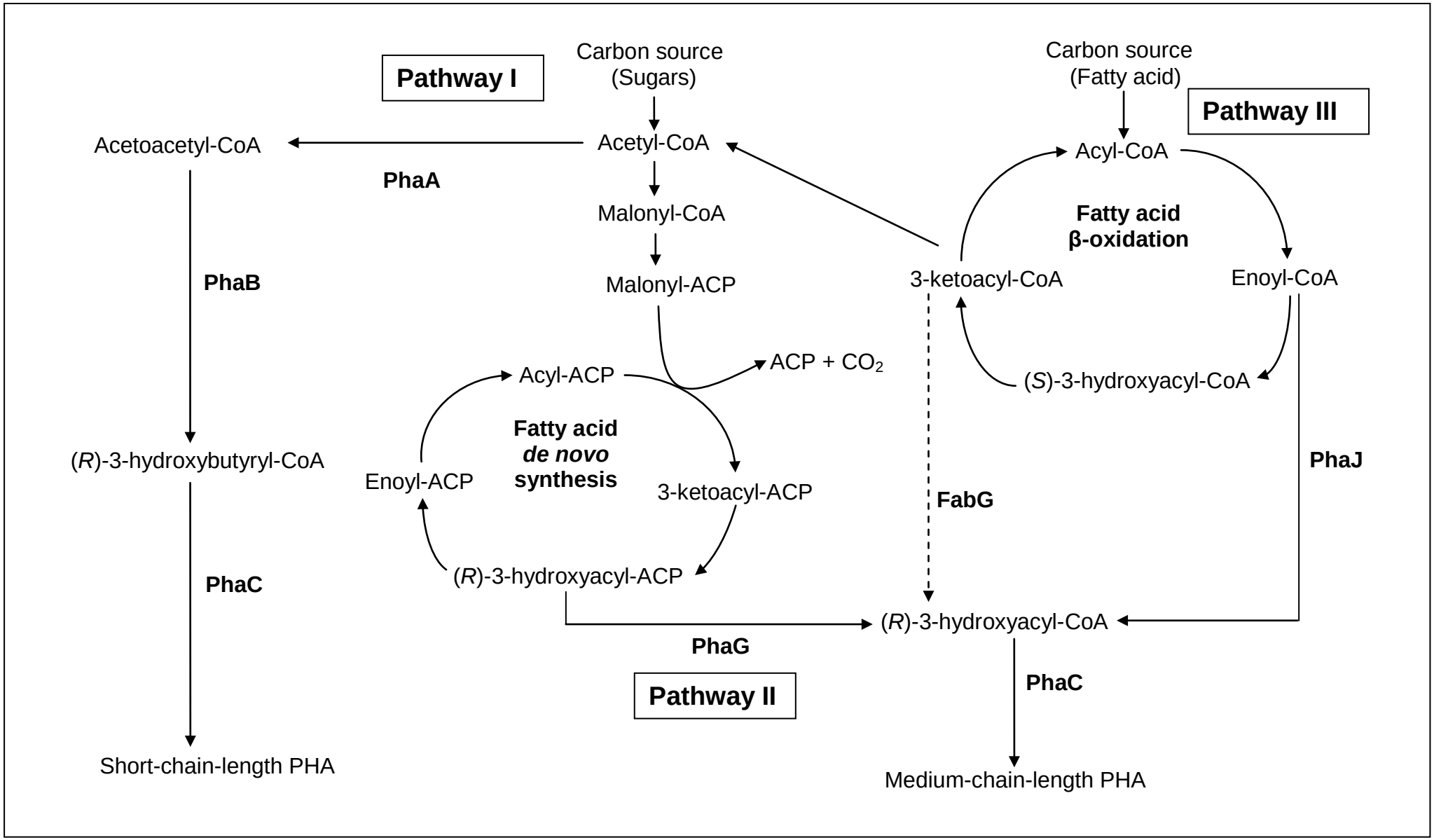


Figure 2.4 Major metabolic routes for PHA biosynthesis (adapted from Rehm, 2007).

ACP-CoA transferase (PhaG) which metabolizes acetyl-CoA and channels it into de novo fatty acid biosynthesis pathway to produce mcl-PHA. PhaG converts (*R*)-3-hydroxyacyl-ACP into (*R*)-3-hydroxyacyl-CoA and then channels the intermediates for PHA synthase to synthesize PHA (Kim *et al.*, 2007; Rehm, 1998).

2.5.2.2 Pathway III

This pathway is also called fatty acid β-oxidation pathway, in which fatty acids or plant oils now serve as the carbon source (Kessler & Witholt, 2001). In this pathway, fatty acids are activated by the acyl-CoA synthetase forming the corresponding acyl-CoA thioesters after uptake into the cell. In this pathway that involves a series of enzymatic steps, (*R*)-specific enoyl-CoA hydratase (PhaJ) plays an imperative role in supplying monomer units for β-oxidation pathway. PhaJ catalyzes (*R*)-specific hydration of the β-oxidation intermediate, *2-trans*-enoyl-CoA into (*R*)-3-hydroxyacyl-CoA which will be polymerized by PHA synthase (Kim *et al.*, 2007; Rehm, 2007).

2.6 CLASSIFICATION OF PHA SYNTHASE

The key enzyme in synthesizing polyhydroxyalkanoates is PHA synthase (PhaC). It determines the type of PHA being synthesized. At present, 88 nucleotide sequences of PHA synthase gene from 68 different bacteria have been recorded. PHA synthase generally can be classified into four major classes based on their primary structures, substrate specificity and also subunit composition as shown in Table 2.2 (Rehm, 2007).

Class I and class II PHA synthases comprise enzymes consisting of only one type subunit, PhaC. The molecular weight (M_w) is between 61 and 73 kDa

Table 2.2 Classification of PHA synthases (adapted from Rehm, 2007).

Class	Subunits	Species	Substrate
I	**PhaC** ~60-73 kDa	*Cupriavidus necator*	$3HA_{scl}$-CoA, $4HA_{scl}$-CoA, $5HA_{scl}$-CoA, $3MA_{scl}$-CoA (~C3-C5)
II	**PhaC** ~60-65 kDa	*Pseudomonas aeruginosa*	3HAmcl-CoA (~≥C5)
III	**PhaC•PhaE** ~40 kDa•~40 kDa	*Allochromatium vinosum*	$3HA_{scl}$-CoA, ($3HA_{mcl}$-CoA [~C6-C8], 4HA-CoA, 5HA-CoA)
IV	**PhaC•PhaR** ~40 kDa•~22kDa	*Bacillus megaterium*	$3HA_{scl}$-CoA

(Qi & Rehm, 2001).

The representative bacteria of class I PHA synthases is *Cupriavidus necator*. The substrates that are preferentially utilized are CoA thioesters of various (*R*)-3-hydroxy fatty acids comprising 3-5 carbon atoms.

Class II PHA synthase is represented by *Pseudomonas aeruginosa*. Pseudomonads normally utilize CoA thioester of various (*R*)-3-hydroxy fatty acids comprising 6-14 carbon atoms as their substrates (Rehm, 2003; Ren *et al.*, 2000).

Class III PHA synthases (represented by *Allochromatium vinosum*), comprise of enzymes consisting two different types of subunits; PhaC subunit and PhaE subunit, both with M_w about 40 kDa. The PhaC subunit possess amino acid sequence similarity from 21%-28% to class I and II PHA synthases, while PhaE subunit exhibit no similarity to PHA synthase. Class III PHA synthases prefer CoA thioesters of (*R*)-3-hydroxy fatty acids comprising 3-5 carbon atoms (Liebergesell *et al.*, 1992; Liebergesell & Steinbuchel, 1992).

Class IV PHA synthases are similar to the class III PHA synthases. Class IV PHA synthases genes are found in a bacteria cluster under the genus *Bacillus*. *Bacillus megaterium* is the classic example of bacteria containing class IV PHA synthase. The only differences between class III and class IV PHA synthases is that the subunit PhaE subunit is replaced by PhaR (M_w about 20 kDa) (McCool & Cannon, 1999; McCool & Cannon, 2001).

However, there are a few exceptions to this classification based on substrate specificity. The PHA synthase of *Thiocapsa pfennigii* contains two different subunits with strong similarity of PhaC subunit to class III PHA synthases. The *T. pfennigii* PHA synthase have a very broad substrate specificity comprising CoA thioesters of scl-PHA and also mcl-PHA (Liebergesell *et al.*, 2000). *Aeromonas punctata* own a

PHA synthase that contain one type of subunit with strong similarity to class I PHA synthases and catalyzes synthesis of a copolymer of P(3HB-co-3HHx) (Fukui & Doi, 1997). The *Pseudomonas* sp. 61-3 possesses PhaC1 and PhaC2 with strong similarity to class II PHA synthase and are able to catalyze the polymerization of a copolymer P(3HB) and mcl-PHA (Matsusaki *et al.*, 1998).

2.7 GENETIC ORGANIZATION OF PHA GENES

PHA biosynthesis genes often appear as a cluster of genes in the bacterial genome. In the well studied bacteria, *C. necator*, genes encoding PHA synthase (*phaC*), β-ketothiolase (*phaA*) and acetoacetyl-CoA reductase (*phaB*) are organized in a *phaCAB* operon (Peoples & Sinskey, 1989a; Peoples & Sinskey, 1989b; Schubert *et al.*, 1988; Slater *et al.*, 1988). Some other PHA accumulating bacteria show a different gene organization but normally the PHB synthesis gene is co-localized with other PHB biosynthesis genes. Meanwhile, some species like *P. denitrificans* contain other genes related to PHA biosynthesis adjacent to the PHA synthase genes, for example, *phaP* (encoding phasin) and *phaR* (encoding regulator protein) (Rehm, 2007). Figure 2.5 depicts the gene organization of PHA genes in representative bacteria from the four major classes.

The *Pseudomonas* strain displays a different characteristic by having two *phaC* genes, designated *phaC1* and *phaC2*. These two genes are separated by *phaZ* (encoding intracellular depolymerase) (Hoffmann & Rehm, 2004; Hoffmann & Rehm, 2005). *Pseudomonas* can accumulate mcl-PHAs exerting elastomeric properties, and the PHA synthase genes belong to class II PHA synthases. Another gene, *phaD* is located downstream of *phaC2*, but upstream of genes *phaI* and *phaF*, which are transcribed in opposite direction. However, genes encoding enzymes

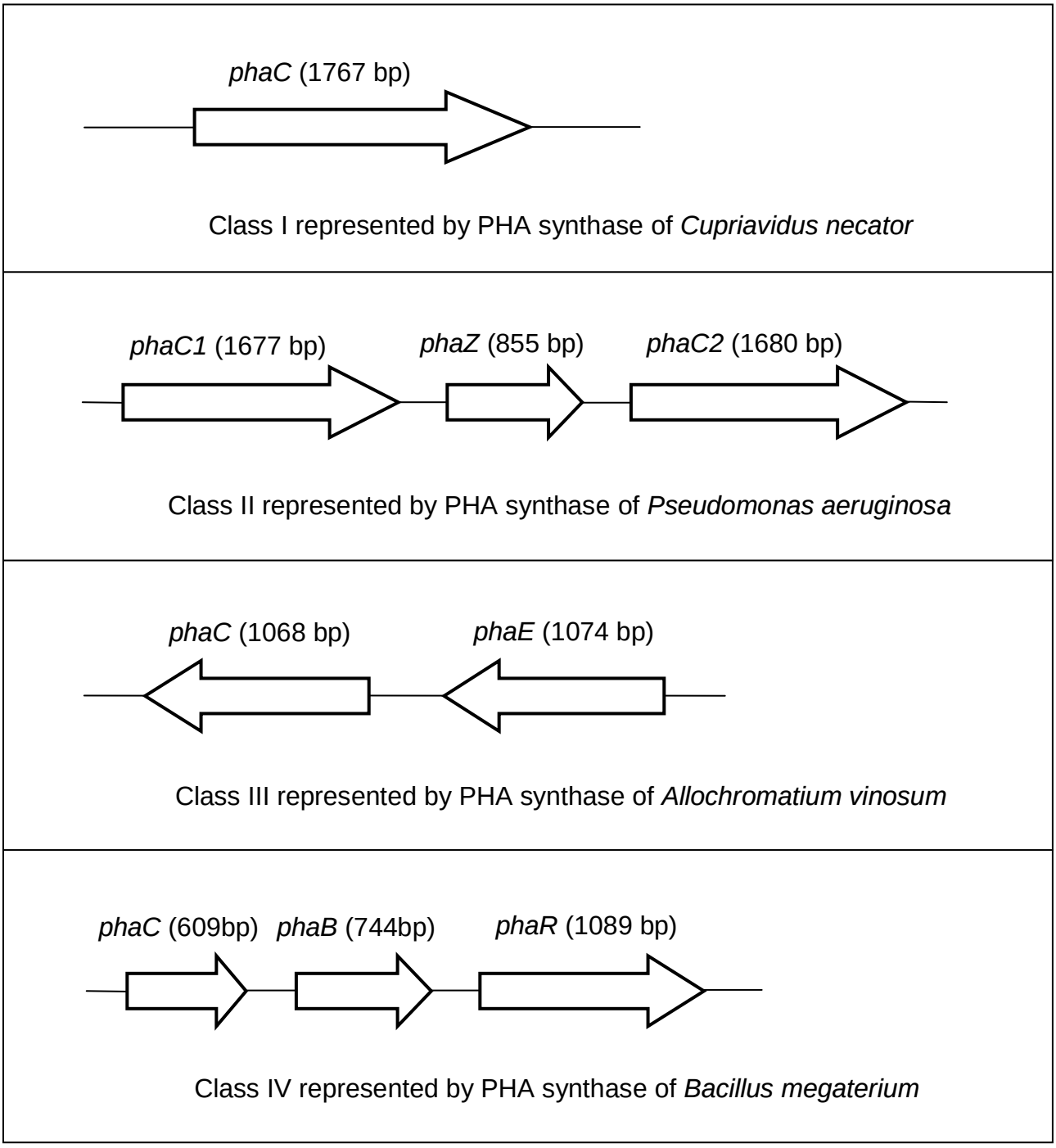


Figure 2.5 Genetic organization of PHA synthase genes in class I, II, III and IV PHA synthases respectively (adapted from Rehm, 2003; Sudesh *et al*., 2000).